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Self-Priming of Retroviral Minus-Strand Strong-Stop DNAs

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After minus-strand strong-stop DNA (−sssDNA) is synthesized, the RNA template is degraded by the RNase H activity of the reverse transcriptase (RT), generating a single-stranded DNA. The 3′ end of −sssDNA from HIV-1 can form a hairpin; this hairpin will self-prime *in vitro.* We previously used a model substrate, −R ssDNA, which corresponds to the 3′ end of the −sssDNA of HIV-1, to show that the self-priming of this model substrate could be prevented by annealing a 17-nt-long DNA oligonucleotide to the 3′ end of −R ssDNA in the presence of HIV-1 nucleocapsid (NC) protein. Similar model substrates were prepared for HIV-2 and HTLV-1; the R regions of these two viruses are longer and form more complex structures than the R region of the HIV-1 genome. However, the size of the R region and the complexity of the secondary structures they can form do not affect self-priming or its prevention. The efficiency of the self-priming is related to the relative stabilities of the conformations of −R ssDNA that can and cannot induce self-priming. For the three viruses (HIV-1, HIV-2, and HTLV-1), the size of the DNA oligonucleotide needed to block self-priming in the presence of NC is similar to the expected size of the piece of RNA left after degradation of the RNA template during reverse transcription. We also found that when the 3′ end of −R ssDNA is annealed to a complementary DNA oligonucleotide, it is a good substrate for efficient nonspecific strand transfer to other single-stranded DNA molecules.

Key Words: self-priming; reverse transcriptase; nucleocapsid protein.

INTRODUCTION

Retroviral reverse transcriptases (RTs) synthesize minus-strand strong-stop DNA (−sssDNA) using the RNA genome as a template. DNA synthesis is initiated from a primer tRNA bound to the primer-binding site (PBS) (Coffin *et al.,* 1997; Goff, 1990; Whitcomb and Hughes, 1992). To synthesize −sssDNA, RT copies the U5 and R regions at the 5′ end of the genome. The synthesis of −sssDNA creates an RNA/DNA heteroduplex; the RNase H activity of RT degrades the RNA in this heteroduplex (Hughes *et al.,* 1996; Palaniappan *et al.,* 1996; Schatz *et al.,* 1990). The nascent single-stranded DNA is then transferred to the R region at the 3′ end of the RNA genome and minus-strand DNA synthesis continues (Coffin, 1979; Kulpa *et al.,* 1997; Panganiban and Fiore, 1988).

The R region of human immunodeficiency virus type 1 (HIV-1) genomic RNA [GenBank Accession No. GI 328415, positions 455 to 550 (Adachi *et al.,* 1986)] contains two large hairpins, the poly(A) and TAR-1 hairpins (Berkhout *et al.,* 1989; Muesing *et al.,* 1987). *In vitro,* nascent HIV-1 −sssDNA can form secondary structures that induce self-priming (Driscoll and Hughes, 2000; Driscoll *et al.,* 2001; Guo *et al.,* 1997). If self-priming occurs, strand transfer is prevented (Guo *et al.,* 1997). HIV-1 nucleocapsid (NC), a 55-amino-acid-long protein that contains two zinc fingers, is synthesized as part of the Gag polyprotein. HIV-1 NC can prevent self-priming of −sssDNA *in vitro* (Driscoll and Hughes, 2000; Guo *et al.,* 1997) and also promotes strand transfer (Lapadat-Tapolsky *et al.,* 1995; Li *et al.,* 1996; Rein *et al.,* 1998). Recently, it was shown that HIV-1 NC and a 17-base-long DNA oligonucleotide complementary to the 3′ end of a model substrate that corresponds to the 3′ end of the −sssDNA of HIV-1 are sufficient to prevent self-priming in an *in vitro* reaction (Driscoll and Hughes, 2000).

The R regions of human immunodeficiency virus type 2 (HIV-2) [GenBank Accession No. GI 1332361, positions 1 to 173 (Clavel *et al.,* 1986)] and human T-cell leukemia virus type 1 (HTLV-1) [GenBank Accession No. GI 221866, positions 354 to 581 (Malik *et al.,* 1988)] are much longer than the R region of HIV-1 (173, 228, and 96 nucleotides, respectively). In the case of HIV-2, it was proposed that this region of the genomic RNA contains three loops that form the transactivation response element (TAR-2) (Emerman *et al.,* 1987; Fenrick *et al.,* 1989). This structure prediction was confirmed by RNase susceptibility measurements (Berkhout, 1992). Similar studies performed on the genome of HTLV-1 demonstrated that this region forms a complex secondary structure at the 5′ end of the viral RNA, the cis-acting Rex-responsive element (RxRE) (Askjaer and Kjems, 1998; Toyoshima *et al.,* 1990).

The secondary structures in −sssDNAs from these two retroviruses might form, at their 3′ ends, secondary structures that induce self-priming. We prepared single-

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stranded DNA model substrates (−R ssDNAs) for HIV-2 and HTLV-1 that are complementary to the R region of the RNA genome. We found that both of these −R ssDNAs can self-prime and the efficiency of the self-priming is dependent on the relative stabilities of the structures that can, and cannot, induce self-priming, not on the size and the complexity of R structures.

After −sssDNA is synthesized, RT degrades the RNA template leaving a 14- to 18-nt-long RNA oligonucleotide annealed to the 3′ end of the nascent DNA strand (Driscoll *et al.,* 2001; Fu and Taylor, 1992). In the three cases that we have studied, a DNA oligonucleotide of a similar size is able to prevent self-priming of −R ssDNA in the presence of HIV-1 NC; longer oligonucleotides can be required in the absence of NC. Thus, the secondary structures at the 3′ end of the −sssDNA that are related to the RNA structures necessary for interactions with regulatory proteins are sufficiently unstable that the piece of RNA left at the 3′ end of −sssDNA is able to prevent self-priming in the presence of NC. The prevention of self-priming does not depend on the stability of the overall structure but only on the stability of the structural element at the 3′ end of −R ssDNA. However, if a DNA oligonucleotide sufficient to block self-priming is annealed to the 3′ end of −R ssDNA, there can be efficient nonspecific strand transfer to single-stranded DNA molecules, even if the single-stranded DNAs are present in the reaction in relatively low concentration.

RESULTS

−R ssDNA secondary structure predictions

A single-stranded DNA (ssDNA) molecule can have different conformations in solution. At equilibrium, the conformation with the lowest free energy (ΔG) will be preferred (the largest portion of the population) and the relative proportion of each of the various possible conformations in the population will be related to the difference in the free energy between the conformations. Because the R region of the RNA genomes of HIV-1 (Berkhout *et al.,* 1989; Muesing *et al.,* 1987), HIV-2 (Berkhout, 1992), and HTLV-1 (Askjaer and Kjems, 1998) contain secondary structure elements (Fig. 1A), the minus-strand DNAs copied from these regions (−R ssDNA) might also form stable secondary structures. However, the thermodynamic parameters governing RNA folding are different from those governing DNA folding, and these differences may affect the conformation and the free energy of the most stable structures. Because the ability of a ssDNA to self-prime is directly linked to its own folding and not to the folding of the complementary RNA molecule, we predicted the possible secondary structures of the −R ssDNAs and their free energies (Fig. 1, Table 1) using mfold version 3.0 software (Materials and Methods). Each possible conformation was categorized according to its ability to self-prime (was the 3′ end double-stranded with another part of the molecule or not?). In the case of HIV-1 −R ssDNA, we found that the most stable structure has a ΔG of −9.8 kcal/mol, but this structure cannot self-prime (the 3′ end is not double-stranded). In the most stable structure that is able to self-prime (the 3′ end is double-stranded) the ΔG is −8.4 kcal/mol. The secondary structure element of the 3′ end that can induce self-priming is the one shown in Fig. 1B. The most stable DNA structure in which the folding of the 3′ end is similar to the folding of the 5′ end of the RNA (the 11 bases at the 3′ end doubled-stranded with bases at positions 114 to 123) has a higher free energy (ΔG of −7.4 kcal/mol). When mutations were introduced in the HIV-1 R sequence to remove three of the mismatches (only the 3-base bulge and a 1-base bubble were retained) as described by Driscoll and Hughes (2000), the hairpin structure in the RNA is much more stable (ΔG of −50.6 kcal/mol). Moreover the hairpin is also the most stable structure in a −R ssDNA containing similar mutations (ΔG of −25.5 kcal/mol). In the case of HIV-2, the most stable structure of the −R ssDNA (ΔG of −24.9 kcal/mol) is not able to self-prime. The most stable structure able to induce self-priming (ΔG of −21.6 kcal/mol) has the 3′ end structure shown in Fig. 1B. The structure in which the folding of the 3′ end of the DNA is similar to the folding of the 5′ end of the RNA is less stable (ΔG of −14.6 kcal/mol). However, the 3′ end of the HIV-2 −R ssDNA, which is the portion of the overall structure involved in the self-priming, is less stable (has more mismatches) than the structures that can induce self-priming of HIV-1 −R ssDNA. For HTLV-1, the most stable DNA structure is able to induce self-priming and the 3′ end of the DNA has a structure similar to the 5′ end of the RNA (stem loop between bases 1 to 25 in the RNA structure) (ΔG of −26.7 kcal/mol). However, in the case of HTLV-1, the structural element involving the very 3′ end of −R ssDNA is relatively small and has several mismatches (Fig. 1B).

According to the structure predictions, we would expect that all these −R ssDNAs could induce self-priming but that HIV-2 −R ssDNA would self-prime less efficiently than either the HIV-1 or the HTLV-1 −R ssDNAs. Moreover, the structural element at the 3′ end of the HIV-1 −R ssDNA that induces self-priming seems to be more stable (size and quality of the basepairing) than the corresponding structures inducing the self-priming of the HIV-2 and HTLV-1 −R ssDNAs.

Nascent HIV-2 and HTLV-1 −R ssDNAs can self-prime

To look at the ability of nascent HIV-2 and HTLV-1 −R ssDNAs to self-prime, R RNAs were synthesized, purified as described (Materials and Methods), and annealed to a 5′ end-labeled DNA oligonucleotide complementary to the 3′ end of the RNA. Then, the complementary DNA

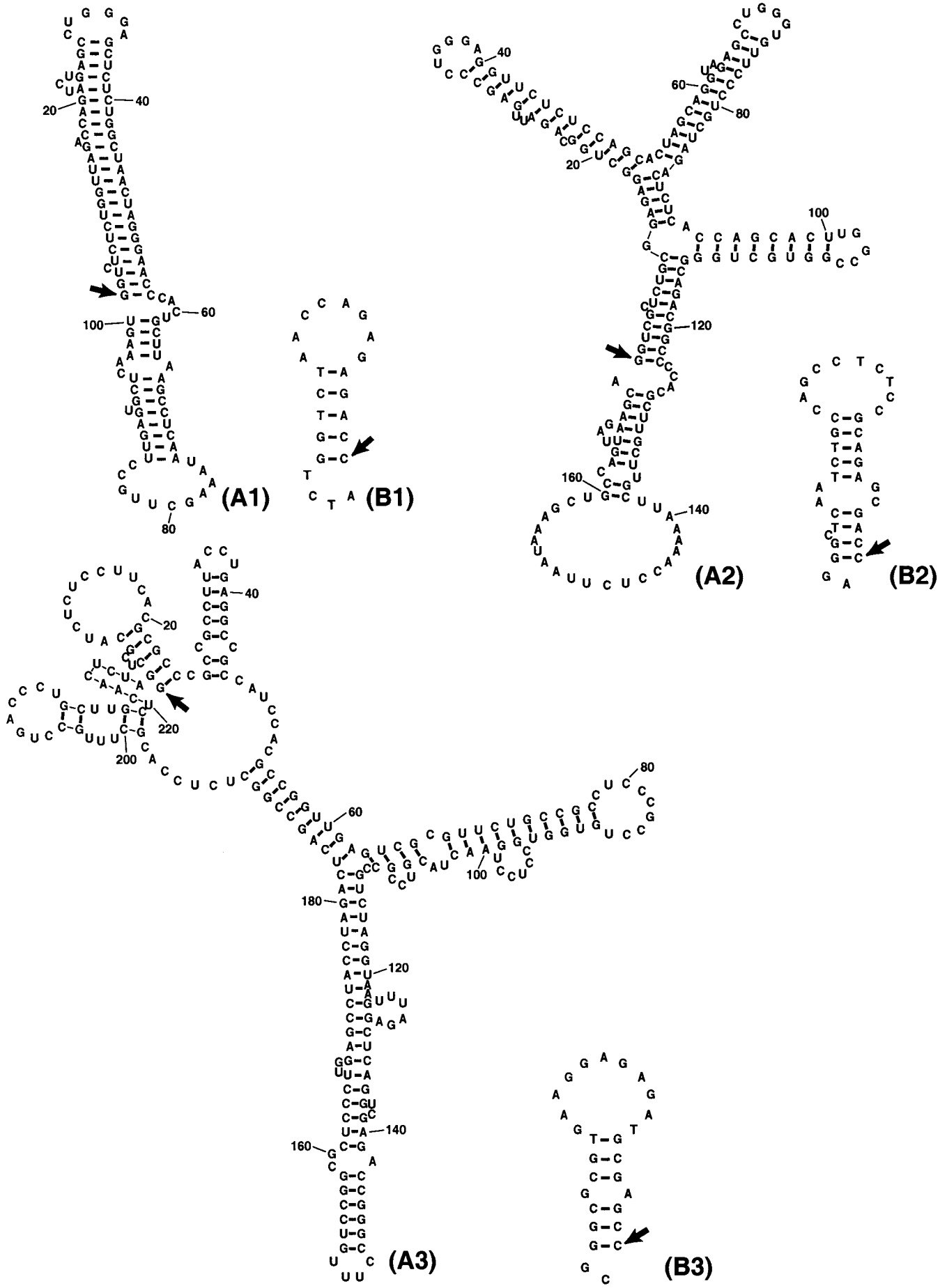
(A1)
(B1)
(A2)
(B2)
(A3)
(B3)

TABLE 1

The ΔG of the Possible Conformations of R RNAs and −R ssDNAs from HIV-1, HIV-2, and HTLV-1

DNA	R size (nt)	Most stable R RNA structure[a]		Most stable −R ssDNA structure[b]		DNA folding inducing self-priming		DNA folding (∼RNA)[c]
		−ΔG[d]	Structure (Fig. 1)	−ΔG[d]	Self-priming[e]	−ΔG[d]	Structure (Fig. 1)[f]	−ΔG[d,g]
HIV-1	96	38.2	A1	9.8	N	8.4	B1	7.4
Modified HIV-1	98	46.5	A1	21.4	Y	21.4	B1	25.5
HIV-2	173	67.3	A2	24.9	N	21.6	B2	14.6
HTLV-1	228	63.4	A3	26.7	Y	26.7	B3	26.7

[a] Calculations were performed at 37°C with [NaCl] = 1 M, [$MgCl_2$] = 0 mM using Zuker algorithm (Materials and Methods).

[b] Calculations were performed at 37°C with [NaCl] = 80 mM, [$MgCl_2$] = 6 mM with oligonucleotide correction using SantaLucia algorithm (Materials and Methods).

[c] Structure with the 3′ end of −R ssDNA forming a secondary structure element similar to what was observed with the 5′ end of R RNA (Fig. 1A).

[d] In kcal/mol.

[e] If the last base at the 3′ end of −R ssDNA is base-paired, then −R ssDNA has the ability to self-prime (Y). If this base is single-stranded, then −R ssDNA has no ability to self-prime in this conformation (N).

[f] Structure showing only the 3′ end of −R ssDNA.

[g] ΔG for a folding of −R ssDNA similar to the folding of R RNA presented in Fig. 1A.

strand (−R ssDNA) was synthesized by HIV-1 RT. Reaction products were analyzed on a denaturing acrylamide gel (Fig. 2A).

In both cases, full-size DNA was synthesized: 173 nt long for HIV-2 (lane 1) and 228 nt long for HTLV-1 (lane 3). Shorter products were also synthesized (data not shown). These products arise from RT pausing at secondary structures of the RNA template (Driscoll *et al.,* 2001). Longer products could also be detected showing that both full-length DNAs can self-prime. In the case of HIV-2, self-primed products typically represented 20 to 30% of the total full-sized DNAs (full-sized or longer); in the case of HTLV-1, more than 60% of the full-length products self-primed. As seen previously with HIV-1 −R ssDNA (Driscoll and Hughes, 2000), the longest self-primed product was shorter than what would be expected if the entire −R ssDNA was copied. We believe that the high level of secondary structure in each of the −R ssDNAs prevented the synthesis of a full-length self-primed product.

Similar experiments were performed in presence of a twofold excess of HIV-1 NC, assuming that each NC molecule covers 7 nucleotides (You *et al.,* 1993). The amount of nucleic acid used in the calculations corresponds to the amount of RNA and primer present at the beginning of the reaction. In fact, the actual NC coating levels varied during the course of the reaction due to polymerization of the DNA strand and degradation of the RNA template. Under these conditions, HIV-1 NC added in twofold excess was able to reduce significantly self-priming of both HIV-2 and HTLV-1 nascent −R ssDNAs (Fig. 2A, lanes 2 and 4). These results clearly show that, in both cases, at least part of the nascent DNA is folded in a manner that can induce self-priming as predicted by thermodynamic studies of −R ssDNA conformations and that, *in vitro,* self-priming of the nascent −R ssDNA can be prevented by the addition of HIV-1 NC, as previously shown with HIV-1 R DNA (Driscoll and Hughes, 2000).

Self-priming of HIV-1, HIV-2, and HTLV-1 −R ssDNAs

It was previously shown (Driscoll and Hughes, 2000) that HIV-1 −R ssDNA can self-prime if incubated with HIV-1 RT. Similar studies were done with HIV-2 and HTLV-1 −R ssDNAs. The −R ssDNAs of HTLV-1 and HIV-2 are too long (228 and 173 nt, respectively) to be synthesized chemically. They were prepared from the corresponding R RNA using SuperScript II RNase H^- reverse transcriptase (Materials and Methods), which is a mutant form of Moloney murine leukemia virus RT (Gerard *et al.,* 1997). Gel purified −R ssDNAs were used in experiments similar to those done previously with HIV-1 −R ssDNA (Driscoll and Hughes, 2000). The experiments were done using the same conditions for HIV-1, HIV-2, and HTLV-1 −R ssDNAs (100 fmol of DNA in 1× RT binding buffer in the presence of 200 fmol of HIV-1

FIG. 1. Secondary structures of HIV-1, HIV-2, and HTLV-1 R RNAs and −R ssDNAs. (A) Secondary structures of R RNAs. (1) The structure of HIV-1 R RNA as proposed by Berkhout *et al.* (1989). (2) The structure of HIV-2 R RNA as proposed by Emerman *et al.* (1987). (3) The structure of HTLV-1 R RNA as proposed by Askjaer and Kjems (1998). The 5′ end is indicated by an arrow. (B) Possible secondary structure elements at the 3′ end of −R ssDNA that could induce self-priming. Calculations were performed using mfold (Materials and Methods) at 37°C with a buffer containing 80 mM NaCl and 6 mM $MgCl_2$. Structures of (1) HIV-1, (2) HIV-2, (3) HTLV-1. The 3′ end is indicated by an arrow.

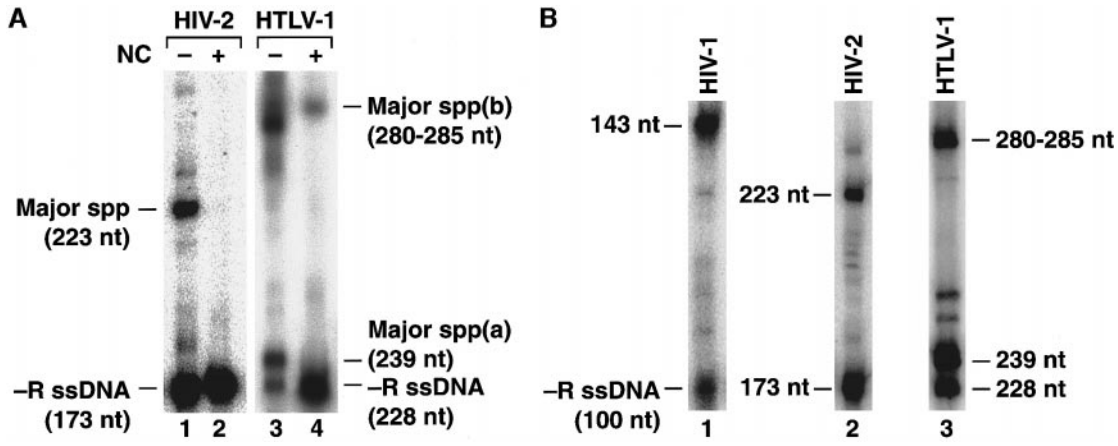

FIG. 2. Self-priming of HIV-1, HIV-2, and HTLV-1 −R ssDNAs. (A) Analysis of nascent HIV-2 and HTLV-1 −R ssDNAs synthesized by HIV-1 RT and effects of HIV-1 NC addition. R RNA was annealed to a ^{32}P-5′-end-labeled primer complementary to the 3′ end of the RNA. The DNA was synthesized by 200 fmol HIV-1 RT for 1 h at 37°C in 1× RT binding buffer and the reaction was analyzed on a 5% denaturing polyacrylamide gel. HIV-2 −R ssDNA without NC (lane 1) and with NC added in twofold excess (lane 2). HTLV-1 −R ssDNA without NC (lane 3) and with NC added in twofold excess (lane 4). The full-size DNA (−R ssDNA) is 173 nt long in the case of HIV-2 and 228 nt long in the case of HTLV-1; spp, self-primed product. (B) Self-priming of HIV-1, HIV-2, and HTLV-1 −R ssDNAs; 100 fmol DNA (internally labeled for HIV-2 and HTLV-1, ^{32}P-5′-end labeled for HIV-1) was incubated in 1× RT binding buffer with 200 fmol HIV-1 RT at 37°C for 1 h. Reaction products were analyzed on a 5% gel. Lane 1, HIV-1; lane 2, HIV-2; lane 3, HTLV-1.

RT at 37°C for 1 h) (Fig. 2B). As expected, all three −R ssDNAs could undergo self-priming and self-primed products were similar to what was seen with the nascent DNAs [Fig. 2A; (Driscoll and Hughes, 2000)]. However, the relative amounts of the different DNA products were, in some cases, modestly different (especially for HTLV-1). As seen with the nascent DNAs, more self-priming occurred with HIV-1 and HTLV-1 −R ssDNAs than with HIV-2 −R ssDNA (50 to 70%, 70 to 80%, and 20 to 30%, respectively, under these conditions).

DNA digestion and analysis on a sequencing gel showed that, in the case of HIV-2 −R ssDNA, the major self-primed product is about 223 nt long (50-base addition). The size of the extension produced by the self-priming reaction corresponds in size to the stem loop between nucleotides 124 and 173 in the RNA structure (Fig. 1A). In the case of HTLV-1, the shortest product is 239 nt long (11-base addition) and there is a second product about 280 to 285 nt long (about a 52- to 57-base addition). In both cases the sizes and the sequences of the major self-primed products are consistent with secondary structures similar to the structures shown in Fig. 1A.

Self-priming can be blocked by a DNA oligonucleotide and HIV-1 NC

When −R ssDNA was made from RNA by HIV-1 RT, HIV-1 NC could prevent self-priming. But when chemically synthesized HIV-1 −R ssDNA was used, HIV-1 NC was able to prevent self-priming only in the presence of a 17-nt-long DNA oligonucleotide complementary to the 3′ end of HIV-1 −R ssDNA (Driscoll and Hughes, 2000). The short DNA oligonucleotide used in these experiments mimics the RNA fragment left after RNase H digestion of the RNA template. In the absence of NC, a 25-nt-long DNA oligonucleotide was able to prevent self-priming of HIV-1 −R ssDNA (data not shown). When mutations were introduced in the HIV-1 −R ssDNA sequence to improve the base-pairing and, consequently, the stability of the hairpin, self-priming was favored and could not be prevented even by the addition of a 21-mer DNA oligonucleotide and HIV-1 NC (Driscoll and Hughes, 2000). Similar experiments were done with HIV-2 and HTLV-1 −R ssDNA (Figs. 3 and 4, Table 2) to see how the size, sequence, and secondary structures of −R ssDNA affect what is required to prevent self-priming.

DNA oligomers complementary to the 3′ end of the −R ssDNAs were synthesized (HIV-1 block, HIV-2 block, and HTLV block oligonucleotides for HIV-1, HIV-2, and HTLV-1 −R ssDNAs, respectively) (Fig. 3). DNA oligomers were added to the −R ssDNAs in 70-fold excess. The mixture was heated to 65°C and cooled to room temperature. If the DNA oligonucleotide is short, annealing of the 3′ end of −R ssDNA to another part of −R ssDNA is thermodynamically favored relative to the formation of the DNA oligonucleotide/−R ssDNA duplex and, consequently, self-priming is not affected. As the size of the DNA oligonucleotide increases, the free energy of the DNA oligonucleotide/−R ssDNA duplex decreases and formation of the duplex becomes more thermodynamically favored; self-priming is reduced or eliminated. The size of the DNA oligonucleotide needed to abolish self-priming depends on the free energy (and the stability) of the structural element responsible of the self-priming. Thus, the more stable the structural element inducing self-

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5′...CTCCCAGGGCTCAATCTGCCAGCCTCTCCGCAGAGCGACC 3′   HIV-2 -R ssDNA
3′...GAGGGUCCCGAGUUAGACGGUCGGAGAGGCGUCUCGCUGG 5′   HIV-2 R RNA

                                        GTCTCGCTGG      block 10
                                    AGGCGTCTCGCTGG      block 14
                                GAGAGGCGTCTCGCTGG       block 17
                            TCGGAGAGGCGTCTCGCTGG        block 20
                        GGTCGGAGAGGCGTCTCGCTGG          block 22
                    GACGGTCGGAGAGGCGTCTCGCTGG           block 25
                AGTTAGACGGTCGGAGAGGCGTCTCGCTGG          block 30
            TCCCGAGTTAGACGGTCGGAGAGGCGTCTCGCTGG         block 35

5′...CAGGTAAGGCGCGCGGGCGCGTGAAGGAGAGATGCGAGCC 3′   HTLV-1 -R ssDNA
3′...GUCCAUUCCGCGCGCCCGCGCACUUCCUCUCUACGCUCGG 5′   HTLV-1 R RNA
                                    CTACGCTCGG      block 10
                                CTCTCTACGCTCGG      block 14
                            TTCCTCTCTACGCTCGG       block 17
                        CACTTCCTCTCTACGCTCGG        block 20
                    CCGCGCACTTCCTCTCTACGCTCGG       block 25
                CGCGCCCGCGCACTTCCTCTCTACGCTAGG      block 30

5′...GAGAGCTCCCAGGCTCAGATCTGGTCTAACCAGAGAGACC 3′   HIV-1 -R ssDNA
3′...CUCUCGAGGGUCCGAGUCUAGACCAGAUUGGUCUCUCUGG 5′   HIV-1 R RNA
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FIG. 3. Sequences of the 40 bases at the 3′ end of HIV-2, HTLV-1, and HIV-1 −R ssDNAs and at the 5′ end of R RNAs and DNA oligonucleotides used in this paper.

priming is, the longer the DNA oligonucleotide required to prevent self-priming will be.

In the case of HIV-2 −R ssDNA (Fig. 4A), HIV-1 NC (added in twofold excess) was able, by itself, to reduce the intensity of the band corresponding to the major self-primed product (223-nt-long product) by more than

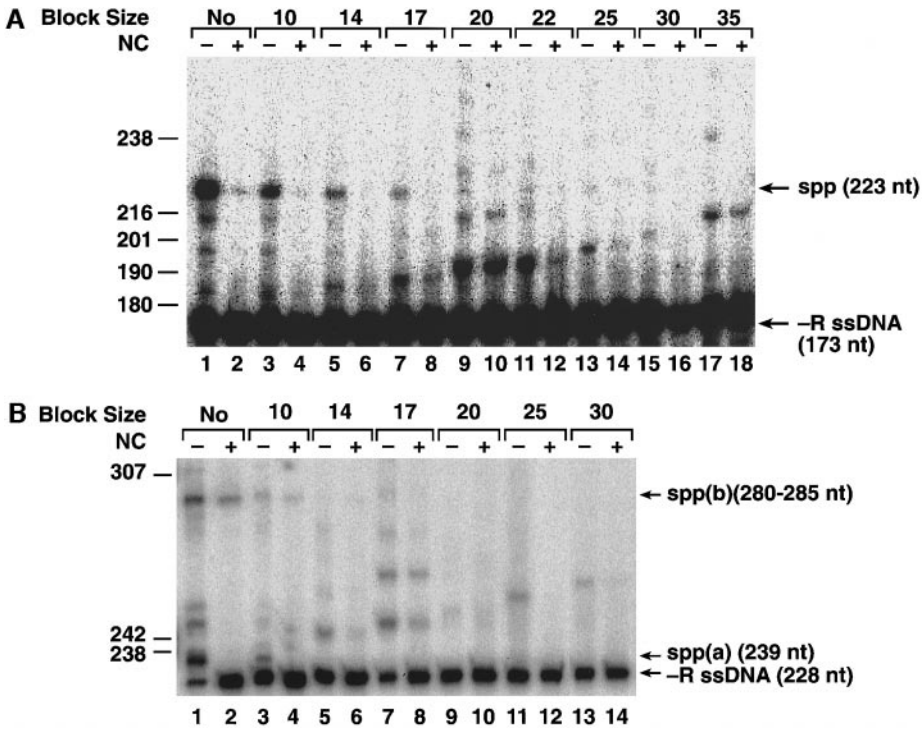

FIG. 4. Effect of a complementary DNA oligonucleotide and HIV-1 NC on −R ssDNA self-priming. (A) Internally labeled HIV-2 −R ssDNA (135 fmol) was incubated with a DNA oligonucleotide (9.5 pmol, 70-fold excess) complementary to its 3′ end at 65°C for 3 min and the solution was cooled to room temperature. In the reactions containing NC, the protein was added in twofold excess, and the reaction tube was incubated for 5 min at 37°C. The reaction was started by addition of RT start solution and 200 fmol HIV-1 RT and performed in 1× RT binding buffer at 37°C for 1 h. Reaction products were analyzed on a 5% denaturing acrylamide gel. The lowest band corresponds to HIV-2 −R ssDNA (173 nt). Block size is the size (in nt) of the DNA oligonucleotide used to block self-priming. (B) Internally labeled HTLV-1 −R ssDNA (67 fmol) was incubated with a DNA oligonucleotide (4.8 pmol, 70-fold excess) complementary to its 3′ end at 65°C for 3 min and the solution was cooled to room temperature. Similar experiments were performed in 1× RT binding buffer. Reaction products were analyzed on a 4% denaturing acrylamide gel. The lowest band corresponds to −R ssDNA (228 nt). Block size is the size (in nt) of the DNA oligonucleotide used to block self-priming.

90% (lanes 1 and 2). This band (and other bands corresponding to the minor self-primed products observed in lane 1) disappeared if the reaction was done in the presence of NC and a 14-mer DNA complementary to the 3′ end of HIV-2 −R ssDNA (Fig. 4A, lane 6). If the 14-mer was added in the absence of NC protein, self-priming was less efficient but still occurred (Fig. 4A, lane 5). In the absence of HIV-1 NC, no significant self-priming was observed when a complementary DNA at least 20 bases long was added to the reaction (Fig. 4A, lane 9).

In the case of HTLV-1 (Fig. 4B), HIV-1 NC alone reduced the amount of self-priming by about 80%. The shortest product disappeared and the amount of the longest product was significantly reduced. All of the other minor products disappeared completely. Again, if a 14-mer oligonucleotide was added in presence of HIV-1 NC, self-priming was prevented (Fig. 4B, lane 6). However, the same 14-mer could also prevent self-priming in the absence of NC. Despite the fact that HIV-1, HIV-2, and HTLV-1 −R ssDNAs are different in size, structure, stability, and ability to self-prime, the size of the DNA oligonucleotide required to prevent self-priming in presence of NC was similar.

Figures 4A and B show that, in the presence of some of the DNA blocking oligonucleotides, new DNAs were synthesized. Because they were synthesized only when the blocking oligonucleotides were added, they cannot be self-primed products. These new DNAs can easily be seen with HIV-2 −R ssDNA and the HIV-2 block 20 and block 22 oligonucleotides and with HTLV-1 −R ssDNA and the HTLV block 14, block 17, and block 25 oligonucleotides. The size of the new products increased with the size of the added oligonucleotides. For reactions involving HIV-2 −R ssDNA and the HIV-2 block 20 oligonucleotide, DNA digestion and analysis of the products on a high-resolution sequencing gel showed that, relative to HIV-2 −R ssDNA, the size of the novel products was longer by 20 nucleotides or a multiple of 20 (data not shown). The intensity of these bands varied depending on the oligonucleotides used; however, there was no simple relationship between the intensity and the size of the DNA blocking oligonucleotide. Sequencing products from the reaction involving the HIV-2 block 20 oligonucleotide and HIV-2 −R ssDNA showed that the extension products contain sequences complementary to HIV-2 block 20 (data not shown). This means that, in some cases, when the 3′ end of HIV-2 or of HTLV-1 −R ssDNA was annealed to a piece of DNA to prevent self-priming, the 3′ end of the −R ssDNA was able to strand transfer to the 3′ end of a single-stranded DNA present in the reaction. Similar results were obtained with HIV-2 RT and HIV-2 −R ssDNA (data not shown).

TABLE 2

Size of the Smallest DNA Blocking Oligonucleotide Able to Prevent Self-Priming of HIV-1, HIV-2, and HTLV-1 −R ssDNAs

−R ssDNA	Oligonucleotide alone (nt)	Oligonucleotide (nt) + HIV-1 NC (2×)
Improved HIV-1	>21[a]	>21[a]
HIV-1	25	17[a]
HIV-2	20	14
HTLV-1	14	14

[a] From Driscoll and Hughes (2000).

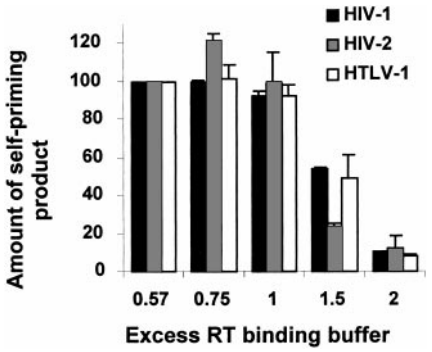

FIG. 5. Salt effects on self-priming of −R ssDNA; 300 fmol of −R ssDNA (internally labeled for HIV-2 and HTLV-1, 5′-end-labeled for HIV-1) was incubated in the presence of 200 fmol HIV-1 RT and RT start solution for 1 h at 37°C in the presence of different amounts of RT binding buffer. Reactions were analyzed on a 5% (HIV-1, HIV-2) or 4% (HTLV-1) denaturing acrylamide gel. For each reaction, the amount of product and the total amount of DNA were quantitated using ImageQuant software. The amount of product was expressed as a percentage of total DNA present in the reaction. For each DNA template, the results were then normalized to the results obtained with the lowest salt concentration (0.5× RT binding buffer).

Salt dependence of the self-priming

To study the salt dependence of the self-priming event, the reactions were performed in the presence of different amounts of RT binding buffer (Fig. 5). The self-priming of HIV-1 and HTLV-1 −R ssDNAs have a similar dependence on salt concentration. In these two cases, the amount of product was not affected by salt concentrations lower than 80 mM (1× RT binding buffer). At higher salt concentrations, the amount of self-primed product was reduced. This suggests that the observed salt effects are probably RT related (polymerization and strand displacement activities) and not related to folding and/or secondary structures of the DNA. HIV-2 −R ssDNA self-priming showed a different salt dependence. For HIV-2 −R ssDNA, the formation of the self-primed product was slightly more efficient in the presence of 60 mM KCl (0.75× RT binding buffer) than in the presence of 46 mM (0.57×) or 80 mM (1×) KCl. The efficiency of the self-priming of HIV-2 −R ssDNA was dramatically reduced at higher salt concentrations. This was not what was observed with HIV-1 and HTLV-1 −R ssDNAs, which suggests a different mechanism; it is likely that some of the effects of salt are due to changes in the distribution

among the conformations of the HIV-2 −R ssDNA that can and cannot self-prime.

Effects of the concentration of the HIV-2 block 20 oligonucleotide on the efficiency of the strand transfer of HIV-2 −R ssDNA

In the previous experiments, a large excess (70-fold) of the DNA blocking oligonucleotide was used in the annealing reaction. Consequently, a large amount of oligonucleotide was free in solution after annealing. To determine whether the free oligonucleotide (HIV-2 block 20 oligonucleotide) affected the strand transfer, the annealing reaction was fractionated on a G-50 spin column to remove the free oligonucleotides, and then the purified −R ssDNA annealed to the HIV-2 block 20 oligonucleotide was used in a self-priming experiment (Fig. 6A, lane 2). A small amount of the band corresponding to the major self-primed product was present, showing that there was a small amount of −R ssDNA that was able to self-prime. Either a small amount of −R ssDNA never annealed to the HIV-2 block 20 oligonucleotide or a small amount of the HIV-2 block 20 oligonucleotide dissociated from the −R ssDNA during or after the purification. The strand transfer product for the HIV-2 block 20 oligonucleotide was present in the control (no G-50 purification, Fig. 6A, lane 9) but was not produced in the reaction that has no free oligonucleotide. These results indicate that the strand transfer to HIV-2 block 20 oligonucleotide is an intermolecular process.

After fractionation of the annealing reaction on a G-50 spin column, variable amounts of the HIV-2 block 20 oligonucleotide were added to a series of polymerization reactions (Fig. 6A, lanes 3 to 8). The intensity of the bands corresponding to the strand transfer products increased as the concentration of the free HIV-2 block 20 oligonucleotide increased. These results showed that there was a direct dependence of the amount of strand transfer product on the amount of free oligonucleotide. However, strand transfer occurred even if a relatively low amount of free HIV-2 block 20 oligonucleotide was present in the reaction (onefold excess, Fig. 6A, lane 3).

We found that the strand transfer reaction can occur with either HIV-2 or HTLV-1 −R ssDNA. In both cases, strand transfer can use different oligonucleotides with different 3′ end sequences as acceptors (Figs. 3 and 4). To see if homology between the DNA primer (−R ssDNA) and the DNA template (free DNA oligonucleotide in solution) is required, an experiment (Fig. 6B) was done with HIV-2 −R ssDNA and the HTLV block 17 oligonucleotide, an oligonucleotide complementary to the 3′ end of the HTLV-1 −R ssDNA (Fig. 3). If the experiment was performed in the presence of HTLV block 17 oligonucleotide, self-priming occurred because the HTLV block 17 oligonucleotide could not anneal to the 3′ end of HIV-2 −R ssDNA (Fig. 6B, lane 1). HIV-2 block 20 oligonucleotide

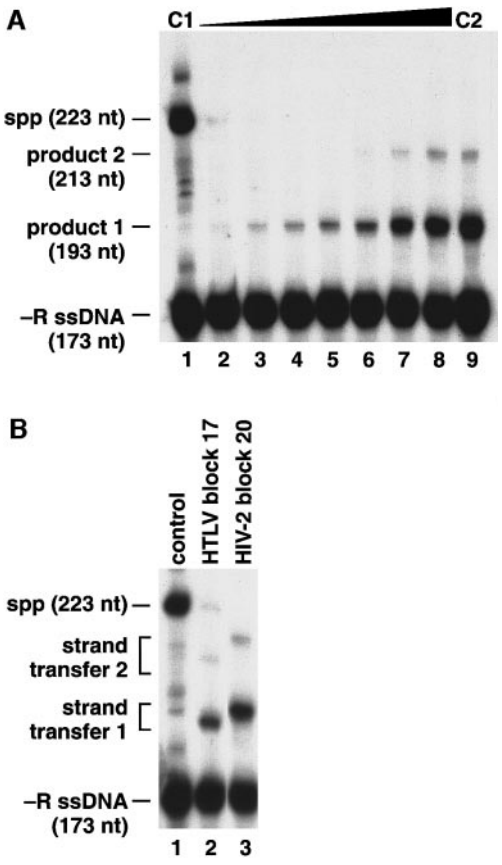

FIG. 6. If the 3′ end of −R ssDNA was annealed to a complementary DNA oligonucleotide, there could be nonspecific strand transfer. (A) Effect of the amount of HIV-2 block 20 oligonucleotide on strand transfer. Lane 1, 200 fmol of internally labeled HIV-2 −R ssDNA was incubated in 0.7× RT binding buffer for 1 h at 37°C in the presence of RT start solution and 200 fmol HIV-1 RT. Lanes 2–8, 200 fmol of internally labeled HIV-2 −R ssDNA was annealed to HIV-2 block 20 oligonucleotide added in 70-fold excess, and then free oligonucleotide was removed by fractionation of the mixture on a G-50 spin column equilibrated with 1× RT binding buffer. Various amounts of HIV-2 block 20 oligonucleotide were added and the reactions were performed in 0.7× RT binding buffer for 1 h at 37°C in the presence of RT start solution and 200 fmol of HIV-1 RT. The reactions were analyzed on a 5% denaturing polyacrylamide gel. Lane 2, no HIV-2 block 20 oligonucleotide was added. Lane 3, the HIV-2 block 20 oligonucleotide was added in onefold excess (by comparison to the number of template molecules). Lane 4, in threefold excess. Lane 5, in 5-fold excess. Lane 6, in 25-fold excess. Lane 7, in 50-fold excess. Lane 8, in 75-fold excess. Lane 9, similar experiment but without G-50 purification after the annealing reaction. The lowest band corresponds to HIV-2 −R ssDNA (173 nt). (B) HIV-2 −R ssDNA strand transfer to the HTLV block 17 oligonucleotide. Lane 1, 200 fmol of internally labeled HIV-2 −R ssDNA was annealed to the HTLV block 17 oligonucleotide (added in 70-fold excess) and then incubated in the presence of 200 fmol of HIV-1 RT for 1 h in 0.7× RT binding buffer at 37°C. Lane 2, 200 fmol on internally labeled HIV-2 −R ssDNA was annealed to HIV-2 block 20 oligonucleotide (added in 70-fold excess) and then the mixture was fractionated on a G-50 spin column. The HTLV block 17 oligonucleotide was added in 70-fold excess and the reaction was performed for 1 h at 37°C in the presence of RT start solution and 200 fmol of HIV-1 RT in 0.7× RT binding buffer. Lane 3, 200 fmol of HIV-2 −R ssDNA was annealed to HIV-2 block 20 oligonucleotide (added in 70-fold excess) and the reaction was performed for 1 h at 37°C in the presence of RT start solution and 200 fmol of HIV-1 RT.

was annealed to HIV-2 −R ssDNA, the annealing reaction was fractionated on a G-50 spin column, and the HTLV block 17 oligonucleotide was added in a 70-fold excess (Fig. 6B, lane 2). As expected, the HIV-2 block 20 oligonucleotide prevented the self-priming and strand transfer products were produced. The products of this reaction were compared to the products obtained when the strand transfer was done in presence of the HIV-2 block 20 oligonucleotide (Fig. 6B, lane 3). It can be seen in the reaction containing HTLV block 17 oligonucleotide that the strand transfer product was smaller than the products of the reaction containing HIV-2 block 20 oligonucleotide. Thus, HTLV block 17 oligonucleotide was the DNA acceptor. These experiments clearly showed that strand transfer requires that the 3′ end of the strand transfer donor DNA must be annealed to a complementary piece of DNA and that the strand transfer can occur even with a strand transfer acceptor which has no significant homology with the DNA donor.

DISCUSSION

When the HIV-1 genome is copied *in vitro,* the nascent −sssDNA is able to self-prime (Driscoll and Hughes, 2000; Guo *et al.,* 1997; Lapadat-Tapolsky *et al.,* 1997). If self-priming occurs *in vivo,* full-length genomic DNA cannot be synthesized. The R regions of HIV-1, HIV-2, and HTLV-1 RNAs contain secondary structures (TAR-1, TAR-2, and RxRE, respectively) (Askjaer and Kjems, 1998; Berkhout, 1992; Muesing *et al.,* 1987). To see if the corresponding −R ssDNAs can fold into structures that can self-prime, secondary structure predictions were performed. The minus-strand DNAs from the R regions of HIV-1, HIV-2, and HTLV-1 have several possible conformations. In the case of HTLV-1, the most stable of the predicted structures can self-prime; in the cases of HIV-1 and HIV-2 −R ssDNAs, secondary structures that should be able to self-prime are not the most energetically favored. As might be expected from these data, HTLV-1 −R ssDNA self-primes more efficiently than HIV-1 and HIV-2 −R ssDNAs. Moreover, when mutations were introduced in the HIV-1 TAR-1 sequence to remove most of the mismatches and stabilize the hairpin, self-priming was enhanced (Driscoll and Hughes, 2000). With all these −R ssDNAs (Driscoll and Hughes, 2000; this paper), the efficiency of self-priming increased with the relative stability of the structures that could induce self-priming. The most important factor affecting the ability of a given DNA to self-prime is the stability of the structures that can self-prime relative to the stability of the structures that cannot self-prime. Thus, the distribution of the −sssDNA molecules among the different possible conformations is governed by the difference in free energy between the structures. The structure with the lowest free energy will be present at the highest concentration. If one of the most stable structures has the 3′ end double-stranded, then self-priming will occur. The efficiency of the self-priming reaction will depend on the difference of free energy between these structures that can self-prime and those that cannot.

When a DNA oligonucleotide complementary to the 3′ end of −R ssDNA is used, annealing will occur only if the energy decrease due to the formation of the oligomer/−R ssDNA duplex is higher than the energy increase due to disruption of the secondary structure element at the 3′ end of −R ssDNA. Consequently, the base-pairing at the 3′ end structural element, the number and the position of bases not involved in canonical Watson–Crick basepairs, and the percentage of G/C basepairs that can be formed will affect the size of the oligomer needed to disrupt the structural element. In the case of HIV-1 −R ssDNA, we calculated the T_m of DNA oligonucleotides complementary to the 3′ end. The shortest DNA oligonucleotide having a T_m higher than the T_m of TAR-1 is 17 nt long (data not shown). In the case of the enhanced TAR-1 hairpin, calculations of the relative energies suggested that the hairpin cannot be disrupted if an oligonucleotide shorter than 23 bases is used (data not shown). In practice, self-priming still occurred in the presence of a 21-mer DNA oligonucleotide even in the presence of NC (Driscoll and Hughes, 2000). HTLV-1 −R ssDNA is longer and has more extensive secondary structures than HIV-1 −R ssDNA but the structure responsible for the self-priming is not the overall R structure but a relatively small stem loop at the 3′ end of −R ssDNA containing only 5 basepairs. In this case, self-priming can be prevented by annealing a DNA oligonucleotide that can disrupt the small and imperfect stem loop involving the 3′ end 25 nucleotides. In the presence of HIV-1 NC, a 10-nt-long DNA significantly reduced the amount of self-priming and a 14-nt-long DNA oligonucleotide completely blocked the formation of the self-primed product. In the case of HIV-2 −R ssDNA, the possible secondary structure elements have relatively poor basepairing at the 3′ end of the −R ssDNA (this structure contains both mispairs and 1-base bulges) and a 14-nt-long DNA oligonucleotide is able to prevent self-priming in the presence of NC.

In the most stable structure, each of the individual structural elements contributes to the decrease in the free energy of the R structure. Consequently, the secondary structure with the overall lowest energy may have a structural element at the 3′ end that is relatively unstable. Thus, in the case of HTLV-1 −R ssDNA, for example, self-priming is favored because the most stable structure has a double-stranded 3′ end. However, the 3′ end secondary structure elements are not particularly stable and, consequently, can be disrupted relatively easily.

Even in the absence of HIV-1 NC, self-priming of −R ssDNA can be prevented by annealing a DNA oligonucleotide. If NC is absent, the oligonucleotide must either be similar in size (HTLV-1) or larger (HIV-1, HIV-2) than in

the presence of NC. These results clearly show that HIV-1 NC is not always required to block self-priming with a DNA oligonucleotide as previously proposed (Driscoll and Hughes, 2000) but that NC can reduce the size of the oligonucleotide necessary to block self-priming.

With HIV-1 −R ssDNA, the size of the oligonucleotide required to block the self-priming in the presence of NC was similar to the size predicted by thermodynamic calculations. The fact that the experimental results are in agreement with the thermodynamic calculations suggests that the presence of a twofold excess of NC does not significantly affect the thermodynamics of the system. However with either HTLV-1 or HIV-2 −R ssDNA, NC can, by itself, reduce self-priming by more than 50%. When similar experiments were performed with HIV-1 −R ssDNA, the effect was more modest (a reduction of approximately 10% in the amount of the self-primed product). There are two simple explanations. First, NC could slow the rate of the polymerization. If the polymerization rate was affected, we would expect to see a similar level of inhibition with all three −R ssDNAs. This explanation is not consistent with the fact that there is a much larger effect on self-priming of HIV-2 and HTLV-1 −R ssDNAs than HIV-1 −R ssDNA (Driscoll and Hughes, 2000; this paper). Second, NC could affect directly the ability of a −R ssDNA to self-prime. It has been suggested that nucleic acid chaperone activity of HIV-1 NC destabilizes secondary structures in RNA and ssDNA and, consequently, facilitates the progression of RT through the genome (Khan and Giedroc, 1992; Tsuchihaschi and Brown, 1994). We found that it was easier to prevent the self-priming of HIV-2 and HTLV-1 −R ssDNAs than HIV-1 −R ssDNA by annealing a DNA oligonucleotide in the absence of NC. This suggests that it is easier to destabilize the structural element formed from the 3′ end of the HIV-2 and HTLV-1 −R ssDNAs than HIV-1 −R ssDNA (the change in free energy due to the formation of structural element at the 3′ end is smaller for HIV-2 and HTLV-1 −R ssDNAs than for HIV-1 −R ssDNA). If the change of free energy produced by NC binding is similar for the three −R ssDNAs, then we could expect a greater effect on the self-priming of HIV-2 and HTLV-1 −R ssDNAs than for HIV-1 −R ssDNA. If the difference of free energy between conformations that can self-prime and those that cannot self-prime is large (for example, in the modified form of HIV-1 −R ssDNA), the small changes that NC may introduce into the thermodynamics of the system might be too modest to affect the distribution between conformations and, consequently, to affect self-priming. But, when this difference of energy between conformations is small (HIV-2 and HTLV-1 −R ssDNAs, for example), the distribution between conformations could be quite sensitive to NC. A simple interpretation of our results (decrease of self-priming in presence of NC) would be that NC destabilizes double-stranded DNA as proposed previously by others (Khan and Giedroc, 1992; Tsuchihaschi and Brown, 1994) and changes the distribution of the molecules in the various possible conformations.

Even though these three retroviruses have R regions that differ significantly in size and overall secondary structure, in every case the self-priming of −R ssDNA can be prevented by a 14- to 17-nt-long DNA oligonucleotide and HIV-1 NC (Driscoll and Hughes, 2000; this paper). However, in some cases like HIV-1 and HIV-2 −R ssDNAs, a much longer DNA oligonucleotide is required to block self-priming in absence of NC. Driscoll *et al.* (2001) showed that HIV-1 RT leaves a 14-base-long RNA oligonucleotide annealed to the 3′ end of HIV-1 −sssDNA. RNA fragments of similar size (14 to 18 nt) were formed with avian myeloblastosis virus, Moloney murine leukemia virus (Fu and Taylor, 1992), and Ty1 (Wilhelm *et al.,* 2000) RTs. The size of the RNA oligonucleotide left at the 3′ end of −sssDNA is similar in size to the DNA oligonucleotide needed to prevent the self-priming of −sssDNA in presence of NC. Thus, even when the R region contains relatively large secondary structure elements, the RNA oligonucleotide remaining after RNase H degradation of the RNA template is sufficient to prevent the self-priming of −sssDNA in our experiments. Although there are secondary structures in R regions that are needed for interaction with transactivating proteins [Tax for HIV-1 and HIV-2, Rex with HTLV-1 (Coffin *et al.,* 1997)], the 3′ ends of the −sssDNAs that contain the complement of these RNA structures are sufficiently unstable that the residual RNA oligonucleotide left after degradation of the RNA template by RT is long enough to prevent self-priming of the −sssDNAs. Thus, sequences found at the 5′ end of some retroviruses have been optimized to form RNA secondary structures necessary for interactions with other retroviral proteins. However, successful reverse transcription requires that the structures at the 3′ end of the −sssDNA be sufficiently unstable that self-priming can be prevented by the 14- to 18-nucleotide-long RNA fragment derived from the 5′ end of the genome by RNase H digestion.

We also found that if the 3′ end of −R ssDNA is annealed to a complementary DNA oligonucleotide, it is a good substrate for strand transfer to other ssDNA molecules in solution even when such ssDNA acceptors were present in low concentration. When the −sssDNA synthesis is complete, the RNase H activity degrades the RNA template, leaving a RNA segment annealed to the 3′ end of −sssDNA. At the step just before the first strand transfer, the 3′ end of −sssDNA is hybridized to a short RNA fragment. This is a similar structure to the DNA/DNA hybrid that serves as a strand transfer donor in our *in vitro* assay. If such strand transfer occurred *in vivo,* it would interfere with the normal synthesis of viral DNA. More studies are necessary to understand this nonspe-

cific strand transfer reaction and how this type of reaction is prevented *in vivo.*

MATERIALS AND METHODS

Wild-type HIV-1 RT (p66/p51) and wild-type HIV-2 RT (p68/p68) were expressed in *Escherichia coli* and purified as described previously (Boyer *et al.,* 1994; Hizi *et al.,* 1991). HIV-1 nucleocapsid protein (p7 Zn^{2+} NC) was generously provided by Drs. Robert Gorelick, Louis Henderson, and Larry Arthur (SAIC Frederick). A 30 μM NC solution was prepared by dissolving lyophilized NC in 1× RT binding buffer [50 mM Tris–HCl, pH 8.3, 80 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA)] and 20% glycerol. NC was stored in 4-μl aliquots in 150-μl tubes at −80°C. Fresh aliquots were thawed immediately prior to use.

Oligonucleotides, including the 100-mer used to represent the 5′ end of HIV-1 −sssDNA, were purchased from Gibco BRL. The 5′ end-labeling was performed using [γ-^{32}P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (New England Biolabs). Products were purified on a G-25 spin column (Millipore). Gel imaging and quantitation were performed using a Molecular Dynamics Storm 860 PhosphorImager using ImageQuant software version 5.0.

DNA and RNA secondary structure predictions

DNA and RNA secondary structure predictions were made using mfold version 3.0 software available on the Zuker and Turner web site (http://mfold2.wustl.edu/~mfold). The algorithms are based on published methods for RNA (Mathews *et al.,* 1999; Zuker *et al.,* 1999) and for DNA (SantaLucia, 1998). For the RNA calculations, the conditions were fixed by the program at 1 M NaCl, 0 mM $MgCl_2$, and 37°C. For the DNA, calculations were performed at 37°C with oligomer corrections and 80 mM NaCl, 6 mM $MgCl_2$, which is equivalent to the salt composition of the buffer used in most of the experiments.

R RNA synthesis

For HIV-2 and HTLV-1, R RNAs correspond exactly to the R region of the genomic RNAs (173 and 228 nt, respectively). For HIV-1, R RNA corresponds to the 4 bases at the 5′ end of U5 and 96 bases of the R region. R RNA from HIV-1 was prepared as described previously (Driscoll and Hughes, 2000). DNAs from HIV-2 and HTLV-1 were amplified in a fashion that they would contain a T7 promoter at the 5′ end of the R region. PCRs were done using the Expand High Fidelity PCR system kit (Roche Molecular Biochemicals). Plasmid templates for the PCR reactions were pROD1.12 containing the HIV-2 provirus (a generous gift from Drs. M. Emerman and L. Montagnier) and pUC-LTR containing the *Sma*I-to-*Rsa*I fragment (positions 31 to 681) of HTLV-1 provirus (a generous gift from Dr. D. Derse). One oligonucleotide used in the amplification contained a modified T7 promoter and the 5′ end of the R region (5′-TTACGCCAAGCTACGTAATACGACTCACTATAGGTCGCTCTGCGGAGAGGCTGGCAGATTG for HIV-2 and 5′-TTACGCCAAGCTACGTAATACGACTCACTATAGGCTCGCATCTCTCCTTCACGCGCCCG for HTLV-1). The second oligonucleotide was complementary to the 3′ end of R (5′-TGCTTCTAACTGGCAGC for HIV-2 and 5′-TAGAGTTGAGCAAGCAGGGTC for HTLV-1). The PCR products were gel purified and used as templates for RNA synthesis with the MEGAshortscript kit (Ambion) according to the manufacturer's instructions. After RNA synthesis, the double-stranded DNA template was removed by DNase I digestion and the RNA purified by electrophoresis on a 5% denaturing polyacrylamide gel. The RNA band was visualized under UV and excised. RNA was eluted overnight in the presence of proteinase K. After phenol extraction and precipitation, RNA was quantitated by absorbance at 260 nm.

Internally labeled −R ssDNA synthesis

R RNA was used as template for −R ssDNA synthesis. The RNA template was annealed to the oligonucleotide complementary to the 3′ end of the R region in SuperScript II first strand buffer. The complementary DNA strand (−R ssDNA) was synthesized using SuperScript II RNase H^- reverse transcriptase enzyme (Gibco BRL) in the presence of cold dNTPs (160 nmol each except dGTP, 3.2 nmol) and [α-^{32}P]dGTP (Amersham Pharmacia) for 3 h at 42°C according to the manufacturer's instructions. Then the RNA template was removed by RNase A digestion; the DNA was gel purified, eluted, and quantitated as described previously for the RNA.

Reverse transcription assays

RNA templates were mixed with a fivefold molar excess of the ^{32}P-5′-end-labeled oligonucleotide complementary to the 3′ end of the template in 1× RT binding buffer (50 mM Tris–HCl, pH 8.3, 80 mM KCl, 1 mM DTT, 0.1 mg/mL BSA). The mixture was incubated at 65°C for 3 min and allowed to cool to room temperature. Then 10× RT start solution containing dNTPs (80 μM final each) and $MgCl_2$ (6 mM final) was added. For reactions containing NC, the protein was added and the reaction mixture was incubated for 5 min at 37°C. Reactions were started by addition of HIV-1 RT and stopped after 1 h by addition of an equal volume of 90% formamide stop solution containing 1% SDS, 4 μg/ml of DNA plasmid, bromophenol blue, and xylene cyanole. Reactions were heated at 95°C for 5 min and fractionated by electrophoresis on a 4 or 5% denaturing polyacrylamide gel containing 0.5% SDS. The gel was dried under vacuum, exposed to a PhosphorImager screen (Molecular Dy-

namics), and quantitated using ImageQuant software version 5.0.

−R ssDNA self-priming assays

HIV-1 block, HIV-2 block, and HTLV block oligonucleotides are DNA oligonucleotides complementary to the 3′ end of −R ssDNA of, respectively, HIV-1, HIV-2, and HTLV-1 −R ssDNAs (Fig. 3). ^{32}P internally labeled −R ssDNA was mixed with 70-fold excess of the indicated DNA blocking oligonucleotide (when needed) in 1× RT binding buffer. The mixture was heated at 65°C for 3 min and allowed to cool to room temperature. RT start solution and NC protein (when needed) were added and the mixture was incubated for 5 min at 37°C. Reactions were started by addition of RT, performed in the indicated buffer at 37°C, and stopped after 1 h by addition of an equal volume of formamide stop solution. Product analysis was performed as described previously.

−R ssDNA self-priming assays using G-50 spin column

DNA blocking oligonucleotides were annealed to the −R ssDNA template as described previously and unannealed oligonucleotides were removed by fractionating the mixture on a 1-ml G-50-spin column (Millipore) equilibrated with 1× RT binding buffer. Then DNA blocking oligonucleotide (when needed), RT start solution, and RT enzyme were added. The reaction was performed in 0.7× RT binding buffer at 37°C and stopped by addition of an equal volume of formamide stop solution after 1 h. Product analysis was performed as described previously.

Sequencing of reaction products

Sequencing of reaction products was performed in 1× RT binding buffer in the presence of 133 μM each dNTP and 1.6 μM dideoxynucleotide for 1 h at 37°C. Then the DNA was annealed to HIV-2 block 35 oligonucleotide (HIV-2 −R ssDNA, Fig. 3) or HTLV block 30 oligonucleotide (HTLV-1 −R ssDNA, Fig. 3) added in 200-fold excess and digested with *Ban*II (HIV-2) or with *Cfo*I (HTLV-1). Digestion products were analyzed on a 12% denaturing acrylamide gel.

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